INDUCTION OF BLOOD VESSEL FORMATION THROUGH ADMINISTRATION OF POLYNUCLEOTIDES ENCODING SPHINGOSINE KINASES

This application is a divisional of U.S. Application No. 09/970,516, filed October 4, 2001, which claims the benefit under 35 USC §119(e) of U.S. Provisional Application No. 60/238,230, filed on October 5, 2000, for "Induction of Blood Vessel Formation Through Administration of Polynucleotides Encoding Sphingosine Kinases." The disclosure of that application is incorporated hereby by reference in its entirety.

This invention relates to the induction of blood vessel formation. More particularly, this invention relates to the induction of blood vessel formation in an animal by administering to the animal a sphingosine kinase, or an analogue, fragment, or derivative thereof. Preferably, the sphingosine kinase, or analogue, fragment, or derivative thereof is administered by administering to the animal a polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof. The polynucleotide encoding the sphingosine kinase may be contained in an appropriate expression vehicle or expression vector, such as an adenoviral vector.

BACKGROUND OF THE INVENTION

Vascular endothelial cells undergo morphogenesis into capillary networks in response to angiogenic factors. It was shown previously that sphingosine-1-phosphate, or SPP, a platelet-derived bioactive lipid, is an important sphingolipid-derived second messenger in mammalian cells that acts to promote proliferation and to inhibit apoptosis. (Olivera, et al., Nature, Vol. 365, pgs. 557-560 (October 7, 1993); Spiegel, et al., J. Leukoc. Biol., Vol. 65, No. 3, pgs. 341-344 (March 1999).) Recently, SPP was defined as a novel regulator of angiogenesis. (Lee, et al., Cell, Vol. 99, No. 3, pgs. 301-312 (October 29, 1999).) SPP activates the endothelial cell differentiation genes (EDG) EDG-1 and EDG-3 subtypes of G protein-coupled receptors on endothelial cells. Both EDG-1 and EDG-3 regulated signaling pathways are required for endothelial cell morphogenesis into capillary-like networks. SPP induces the Gi/mitogen-activated protein kinase cell survival pathway and enhances small GTPase Rho and Rac coupled adherens junction assembly. (Lee, 1999.) The level of SPP is regulated potentially by the enzyme that catalyzes the phosporylation of sphingosine to SPP. The cloning and characterization of the first mammalian sphingosine kinases (murine

SPHK1α and SPHK1β) has been reported. (Kohama, et al., J. Biol. Chem., Vol. 273, No. 37, pgs. 23722-23728 (September 11, 1998)). Human sphingosine kinases (SPHK1 and SPHK2) have also been reported. (Nava, et al., FEBS, 473:81-84 (2000) and Liu, et al., J. Biol. Chem., 275:19513-19520 (2000).)

SUMMARY OF THE INVENTION

Applicants have discovered that the administration of sphingosine kinase, and in particular, that vector-mediated expression of sphingosine kinase enhances the formation of new blood vessels. Thus, the present invention is directed to inducing blood vessel formation in an animal by administering to the animal a sphingosine kinase, preferably by administering to the animal a polynucleotide encoding a sphingosine kinase or an analogue, fragment, or derivative thereof. The sphingosine kinase, or analogue, fragment, or derivative thereof, or polynucleotide encoding sphingosine kinase or an analogue, fragment, or derivative thereof, may be administered in combination with other angiogenic proteins or polynucleotides encoding other angiogenic proteins such as, but not limited to, VEGF, FGF, IGF, angiopoietins, PD-EGF, TGFβ, HIF1-α, nitric oxide synthase, MCP-1, Interleukin-8, ephrins, NAP-2, ENA-78, GROW-2, and fragments of tyrosyl-tRNA synthetase that have angiogenic activity as disclosed in U.S. patent applications 60/193,471 filed March 31, 2000, and 09/813,718, filed March 21, 2001. The polynucleotide encoding the sphingosine kinase may be contained in an appropriate expression vehicle or expression vector, such as a viral vector. The administration of a polynucleotide encoding a sphingosine kinase to an animal is a method by which SPP can be delivered at elevated levels to a local site, and such method provides for the formation of larger vessels containing a well-defined structure that is supported by mural cells such as pericytes and smooth-muscle cells.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention now will be described with respect to the drawings, wherein:

Figure 1 is a map of the plasmid pAv1xsphK1α.

Figures 2A and 2B show the cDNA and amino acid sequences for murine sphingosine kinase 1α.

Figure 3 is a schematic of the adenoviral vector Av3sphK1α.

Figure 4. Hemoglobin assay. Matrigel is snap-frozen with dry ice and dried overnight. The dry weight is recorded and the plug is rehydrated with 0.5 ml of 0.5% Tween and water. After homogenization, the plug is centrifuged at 14,000 rpm for 30 minutes. The

supernatant is collected and its absorbance is read at 405 nm and converted to μg hemoglobin per mg Matrigel using a standard curve generated with hemoglobin standards.

Figure 5 illustrates the remarkable difference that the addition of an adenoviral vector expressing sphingosine kinase makes in the nature of neovessels formed in an *in vivo* matrigel system. Figure 5A shows a hematoxylin and eosin stained section of matrigel treated with S8 cells transduced with an adenoviral vector expressing sphingosine kinase. Figure 5B is the same section stained with α-smooth muscle (α-SM) actin. Figure 5C is a section of a matrigel plug treated with FGF generated capillaries.

Figure 6 illustrates the sphingosine kinase mediated protection of cardiomyocytes.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with an aspect of the present invention, there is provided a method of inducing blood vessel formation in an animal. The method comprises administering to the animal an effective amount of a sphingosine kinase or an analogue, fragment, or derivative thereof.

In accordance with another aspect of the present invention, there is provided a method for the prevention or the treatment of congestive heart failure in an animal. The method comprises administering to said an effective amount of animal a sphingosine kinase, or an analogue, fragment, or derivative thereof.

In accordance with yet another aspect of the present invention, there is provided a method for the prevention or the treatment of myocardial ischemia in an animal. The method comprises administering to said animal an effective amount of a sphingosine kinase, or an analogue, fragment, or derivative thereof.

In accordance with yet another aspect of the present invention, there is provided a method for the prevention or the treatment of ischemia-reperfusion injury in an animal. The method comprises administering to said animal an effective amount of a sphingosine kinase, or an analogue, fragment, or derivative thereof.

In a preferred embodiment, the sphingosine kinase, or analogue, fragment, or derivative thereof is administered to the animal by administering to the animal an effective amount of a polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof. The sphingosine kinase is mammalian, preferably primate, and most preferably human sphingosine kinase. Specific examples of sphingosine kinase amino acid sequences and the polynucleotides encoding them are found in Genbank for human SPHK1 and SPHK2 (accession numbers AF200328 and AF245447), mouse SPHK1α, SPHK1β, and

SPHK2 (accession numbers AF068748, AF068749, and AF245448), and rat SPHK1a, SPHK1c, SPHK1d, SPHK1e, and SPHK1f (accession numbers AB049571, AB049572, AB049573, AB049574, and AB049575). SEQ. ID NO:1 and SEQ ID NO:2 show the cDNA and amino acid sequences for human SPHK1. SEQ ID NO:3 and SEQ ID NO:4 show the cDNA and amino acid sequences for human SPHK2. An analogue of sphingosine kinase includes, but is not limited to, splice variants of sphingosine kinase, deletions in the coding region, and multiple forms (T. Kohama et al., JBC, 273:23722-23728 (1998); H. Liu et al., JBC, 275:19513-19520 (2000); Y. Banno, et al., Biochem J., 335:301-304 (1998)). A fragment of sphingosine kinase is a portion of the protein that retains its activity for inducing blood vessel formation. A derivative of sphingosine kinase includes, but is not limited to, modifications to alter sphingosine kinase regulation or biological activity. Non-limiting examples include the addition of a signal sequence to force secretion of the enzyme or modification of the calcium, calmodulin binding domain, ATP binding site, or membrane retention sequences. The polynucleotide is under the control of a suitable promoter. It is to be understood, however, that the scope of the present invention is not to be limited to any specific promoters.

Preferably, the polynucleotide encoding the sphingosine kinase, or an analogue, fragment, or derivative thereof is contained in an appropriate expression vehicle. Such expression vehicles include, but are not limited to, plasmids, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial vectors), and viral vectors. In one embodiment, the vector is a viral vector. Viral vectors which may be employed include RNA virus vectors (such as retroviral vectors, including lentiviral vectors) and DNA virus vectors (such as adenoviral vectors, adeno-associated virus vectors, Herpes Virus vectors, and vaccinia virus vectors). When a DNA virus vector is employed in constructing the vector, the polynucleotide encoding the sphingosine kinase is in the form of DNA. When an RNA virus vector is employed in constructing the vector, the polynucleotide encoding the sphingosine kinase is in the form of RNA. Preferable viral vectors include adenoviral vectors (preferably lacking all viral genes, i.e. high capacity or gutless), lentiviral vectors (e.g. HIV, BIV-based), and adeno-associated virus (AAV) vectors.

In one embodiment, the viral vector including the polynucleotide encoding sphingosine kinase, or an analogue, fragment, or derivative thereof is an adenoviral vector.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome (Shenk et al., Curr. Top. Microbiol. Immunol., 111(3): 1-39 (1984). Alternatively, the adenoviral vector may be a

modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In a preferred embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; a DNA sequence encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof, and a promoter controlling the DNA sequence encoding a sphingosine kinase, or an analogous, fragment, or derivative thereof. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter. In one embodiment, the vector also is free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences.

In another embodiment, the vector is free of at least the majority of the adenoviral E1 and E3 DNA sequences, and is free of a portion of the other of the E2 and E4 DNA sequences.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at 32°C, the temperature at which the viral particles are produced. This temperature sensitive mutant is described in Ensinger, et al., J. Virology, 10:328-339 (1972), Van der Vliet *et al.*, J. Virology, 15:348-354 (1975), and Friefeld, *et al.*, Virology, 124:380-389 (1983).

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an Ela enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a multiple cloning site (which may be as herein described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The promoter may, in one embodiment, be a regulatable promoter, such as, for example, a glucocorticoid-responsive promoter or an estrogen-responsive promoter, or the promoter may be a tissue – specific promoter. The vector also may, in another embodiment, contain genomic elements which may increase and/or maintain expression of the DNA sequence encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof. Such genomic elements include, but are not limited to, introns, exons, polyadenylation sequences, and 5' and 3' untranslated regions. Such genomic elements, and representative examples thereof, also are described in U.S. Patent No. 5,935,935, issued August 10, 1999. The vector also may contain a tripartite leader sequence. The DNA segment which

corresponds to a segment of the adenoviral genome serves as a substrate for homologous recombination with an adenovirus. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAvS6, which is described in published PCT Application Nos. W0 94/23582, published October 27, 1994, and W0 95/09654, published April 13, 1995, and in U.S. Patent No. 5,543,328, issued August 6, 1996. The DNA sequence encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof then may be inserted into the multiple cloning site of the shuttle plasmid to produce a plasmid vector.

This construct is then used to produce an adenoviral vector. Homologous recombination may be effected through co-transfection of the plasmid vector and the adenovirus into a helper cell line, such as 293 cells, by $CaPO_4$ precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed that includes DNA sequences derived from the shuttle plasmid between the 5'ITR and the homologous recombination fragment, and the DNA derived from the adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the Adenovirus 5 (ATCC VR-5) genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence; a promoter; a DNA sequence encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof; a poly A signal; adenoviral DNA sequences; and an adenoviral 3' ITR. The vector also may include a tripartite leader sequence. The vector may then be transfected into a helper cell line, such as the 293 helper cell line (ATCC No. CRL1573), which will include the E1a and the E1b DNA sequences, which are necessary for viral replication, and to generate adenoviral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes.

In another embodiment, the adenoviral vector is free of all or a portion of each of the adenoviral E1 and E4 DNA sequences, or is free of all or a portion of each of the adenoviral E1 and E2 DNA sequences, or is free of all or a portion of each of the E1, E2, and E4 DNA sequences.

Such vectors may be assembled by direct *in vitro* ligation from combinations of plasmids containing portions of modified or unmodified virus genome or plasmids and fragments derived directly from a linear adenoviral genome, such as the Adenovirus 5

genome (ATCC No. VR-5) or Adenovirus 5 derived viruses containing mutations or deletions.

In another alternative, the vectors can be assembled by homologous recombination, within a eukaryotic cell, between a plasmid clone containing a portion of the adenoviral genome (such as the Adenovirus 5 genome or the adenovirus 5 E3-mutant Add1327 (Thimmapaya, et al., Cell, Vol. 31, pg, 543 (1983)) with the desired modifications, and a second plasmid (such as, for example pAvS6), containing the left adenoviral ITR, an E1 region deletion, and the desired transgene. Alternatively, homologous recombination may be carried out between a plasmid clone and a fragment derived directly from a linear adenovirus (such as Adenovirus 5, or Ad d1327 or an Adenovirus 5 derived virus containing mutations or deletions) genome.

The vector then is transfected into a cell line capable of complementing the function of any essential genes deleted from the viral vector, in order to generate infectious viral particles. The cell line in general is a cell line, which is infectable and able to support adenovirus or adenoviral vector growth and provide for continued virus production. Cell lines which may be transfected with the essential adenoviral genes, and thus may be employed for generating the infectious adenoviral particles include, but are not limited to, the A549, KB, and Hep-2 cell lines.

Because the expression of some viral genes may be toxic to cells, the E1 region, as well as the E2b, and /or E4 regions, may be under the control of an inducible promoter. Such inducible promoters may include, but are not limited to , the mouse mammary tumor virus (MMTV) promoter (Archer, et al., Science, Vol. 255, pgs. 1573-1576 (March 20, 1992)); the synthetic minimal glucocorticoid response element promoter GRE5 (Mader, et al., Proc. Nat. Acad. Sci., Vol. 90, pgs. 5603-5607 (June 1993)); or the tetracycline-responsive promoters (Gossen, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 5547-5551 (June 1992)). In another alternative, the E1 region is under the control of an inducible promoter, and the E2a, E2b and /or E4 regions are under the control of their native promoters. In such alternative, the native promoters are transactivated by expression of the E1 region.

In one embodiment, the cell line includes the entire adenoviral E4 region with its native promoter region, and the E1a region or the entire E1 region (including the E1a and E1b regions) under the control of a regulatable or inducible promoter, such as, for example, the mouse mammary tumor virus (or MMTV) promoter, which is a hormone inducible promoter, or other such promoters containing glucocorticoid responsive elements (GRE's) for transcriptional control. In another embodiment, the E4 DNA sequence also is expressed

from a regulatable promoter, such as the MMTV promoter. The E1 and E4 DNA sequences may be included in one expression vehicle, or may be included in separate expression vehicles. Preferably, the expression vehicles are plasmid vectors which integrate into the genome of the cell line.

Such vectors, wherein the vector is free of all or a portion of each of the adenoviral E1 and E4 DNA sequences, or is free of all or a portion of each of the adenoviral E1 and E2 DNA sequences, or is free of all or a portion of the E1, E2 and E4 DNA sequences, and the complementing cell lines, also are described in PCT Application No. WO96/18418, published June 20, 1996, the contents of which are incorporated herein by reference.

In another embodiment, the adenoviral vector is free of all adenoviral coding regions. This "gutless" adenoviral vector includes an adenoviral 5' ITR, an adenoviral packaging signal, a DNA sequence encoding sphingosine kinase or an analogue, fragment, or derivative thereof, and an adenoviral 3' ITR. The vector contains from about 26 kb to about 38 kb, preferably 28 kb to 32 kb, and may include one or more genomic elements.

The various adenoviral vectors may include promoters other than a sphingosine kinase promoter, such as tissue-specific promoters. The vector also may include, in addition to a DNA sequence encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof, DNA sequences encoding additional proteins which facilitate the generation of new blood vessels, such as, but not limited to, vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), IGFs, angiopoietins, including angiopoietin 1, and angiopoietin 2, TGF-β, hypoxia inducible factors (HIFs) such as HIF1-α, monocyte chemoattractant proteins (MCPs) such as MCP-1, nitric oxide synthase, ephrins, such as ephrin B2, and other angiogenic genes, platelet derived endothelial growth factor, and Interleukin-8.

The adenoviral vector of the present invention may be administered to a host *in vivo* in an amount effective to promote blood vessel formation in an animal host. The host may be a mammalian host, including human and non-human primate hosts.

In one embodiment, the adenoviral vector is administered in an amount from about 10^7 plaque forming units to about 10^{12} plaque forming units, preferably from about $5x10^8$ plaque forming units to about $2x10^{11}$ plaque forming units. Alternatively, cells transduced with the adenoviral vector may be administered in an amount of from about 10^3 to 10^8 cells.

In general, the adenoviral vectors are administered at the local site of ischemia or where therapeutic angiogenesis is required. Delivery can be performed by a variety of means including, but not limited to, direct injection of the adenoviral vector or cells transduced with

the adenoviral vector, intraarterial delivery by a guided catheter or by computer guided systems such as NOGA, or by electroporation.

Alternatively, the adenoviral vector may have a modified fiber protein whereby the adenoviral vector is "targeted" to a specific cell. Representative examples of such adenoviral vectors are disclosed in U.S. Patent No. 5,543,328. Such adenoviral vectors may be administered systemically, such as by intravenous administration (such as, for example, portal vein injection or peripheral vein injection), or intraarterial administration, including hepatic artery administration.

The adenoviral vectors may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, microcarrier beads or localizing agents such as calcitonin gel, hyaluronan solutions, or fibrin plugs derived from the activation of fibrinogen by thrombin (U.S. Patent No. 6,117,425).

In one embodiment, the viral vector is a retroviral vector. Retroviral vectors, including lentiviral viral vectors, have a coding capacity of approximately 8 kb, and the viral genome is capable of integration into the host cell chromosome, thus allowing for potentially life-long transgene expression. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, myeloproliferative sarcoma virus, and mammary tumor virus, as well as lentiviral vectors. One non-limiting example of a lentivirus is Bovine-Immunodeficiency-Virus (BIV). See PCT patent publication WO 01/44458. The vector generally is a replication incompetent retrovirus particle.

In one embodiment, the retroviral vector may be generated from a retroviral plasmid vector which is derived from Moloney Murine Leukemia Virus and is of the LN series of vectors, which are described further in Bender, *et al.*, J. Virol., Vol. 61, pgs. 1639-1649 (1987) and Miller, *et al.*, Biotechniques, Vol. 7, pgs 980-990 (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term “mutated” as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragments or truncations thereof, are not expressed.

In another embodiment, the retroviral plasmid vector may include at least one cloning, or restriction enzyme recognition site, wherein the site(s) has an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction

product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral plasmid vector includes each of these cloning sites. Such vectors are further described in U.S. Patent No. 5,672,510.

When a retroviral plasmid vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral plasmid vector. The shuttle cloning vector also includes a polynucleotide encoding a sphingosine kinase, or an analogue, fragment or derivative thereof which is capable of being transferred from the shuttle cloning vector to the retroviral plasmid vector.

In one embodiment the viral vector is a lentiviral vector, for example derived from BIV. Lentiviral vectors are generally constructed such that the majority of the viral genes are deleted and replaced by a gene of interest. Most frequently the gene of interest is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Alternatively, the gene of interest may be expressed under the regulation of an internal promoter. The genes which have been deleted from the vector are generally provided by one or more helper or packaging constructs in a packaging cell line (Bender et al., J. Virol. 61: 1639 – 1649 (1987) and Miller et al., Biotechniques, 7:980 –990 (1989)). Also see Markowitz et al., J. Virol. 62:1120 – 1124 (1988) wherein complementary portions of the helper construct were divided on two separate constructs. The packaging cell line may be transfected with the retroviral vector, thereby producing vector RNA that is packaged into the virus particles. These released virus particles are replication defective and can be used to deliver the retroviral vector carrying the gene of interest to target cells.

To increase safety, efficiency and accuracy of the recombinant vector systems, various improved recombinant systems have been constructed. One type of improvement includes making safer packaging cell lines that are generated by deletions in the 3' Long Terminal Repeat (LTR). Other improvements include increasing the host range by replacement of one viral env gene with that of another viral env gene thereby creating a hybrid producer line that generates pseudotyped helper viruses. More specifically HIV has been given an extended host cell range by pseudotyping with the unrelated viruses VSV and HSV (Zhu et al., J. Aids, 3:215 – 219 (1990) and Naldini et al., Science, 272:263-267, (1996)). Further improvements have been made by the use of minimum viral coding regions on the vector. Lentivirus can infect nondividing cells, and this property is especially useful

for in vivo gene therapy. Additionally, most packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the necessary coding sequences so that multiple recombination events would be necessary before replication competent virus can be produced. US patents 5,665,577, 5,994,136, 6,013,516 describes examples of lentiviral vector systems. U.S. patent application 09/734,836 and PCT patent publication WO 01/44458 describe examples of BIV based lentiviral vector systems.

In one embodiment, an effective amount of the lentiviral vector is administered to an animal in an amount from about $5x10^5$ transducing units to about 10^{12} transducing units. In a preferred embodiment, the effective amount delivered to the animal is from about $5x10^5$ transducing units to about 10^{10} transducing units.

In one embodiment, in addition to the polynucleotide encoding sphingosine kinase or an analogue, fragment, or derivative thereof, the retroviral plasmid vector also may include polynucleotides encoding additional proteins which facilitate the generation of new blood vessels, such as, but not limited to, insulin like growth factor (IGF), angiopoietins, including angiopoietin1 and angiopoietin2, FGF, nitric oxide synthase, Interleukin-8, and TGF-β.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites are the compatible, or complementary cloning sites herein above described. A polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof and/or a promoter having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

The retroviral plasmid vector may include a promoter for expressing a polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, *et al.*, Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, cyclin G1, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The

selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The retroviral plasmid vector including the polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof is transduced into a packaging cell line including nucleic acid sequences encoding the gag, pol, and env retroviral proteins. Examples of such packaging cell lines include, but are not limited to, the PE501, PA317 (ATCC No. CRL 9078), Ψ-2, Ψ-AM, PA12, T19-14X, VT-19-17-H2, ΨCRE, ΨCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety, or the 293T cell line (U.S. Patent. No, 5,952,225). The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. Such producer cells generate infectious retroviral vector particles which include the polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof.

The retroviral vector particles or cells transduced with a retroviral vector are administered to an animal in an amount which is effective to promote blood vessel formation. Administration of the retroviral vector particles may be by local administration, such as direct injection of the vectors or cells transduced with the vectors, intraarterial delivery by a guided catheter, by computer guided systems such as NOGA and by electroporation. In general, the retroviral vectors are administered in an amount of at least 10^4 cfu/ml, and in general, such an amount does not exceed 10^9 cfu/ml. The exact dosage to be administered is dependent upon a variety of factors including the age, weight, and sex of the animal or patient to be treated, and the disease or disorder being treated.

The retroviral vectors also may be administered in conjunction with an acceptable pharmaceutical carrier, such as, for example, saline solution, protamine sulfate (Elkins-Sinn, Inc., Cherry Hill, N.J.), water, aqueous buffers, such as phosphate buffers and Tris buffers, or Polybrene (Sigma Chemical, St. Louis, MO), or localizing agents such as calcitonin gel, hyaluronan solutions, or fibrin plugs derived from the activation of fibrinogen by thrombin (U.S. Patent No. 6,117,425). The selection of a suitable pharmaceutical carrier is deemed to be apparent to those skilled in the art from the teachings contained herein.

In another alternative, the retroviral vectors hereinabove described, or a polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof, may be encapsulated within liposomes. The liposomes, which encapsulate the retroviral vectors or a polynucleotide encoding sphingosine kinase, or analogue, fragment, or

derivative thereof, may be administered to a host in conjunction with a pharmaceutical carrier as hereinabove described.

In another alternative, retroviral producer cells, such as those derived from the packaging cell lines herein above described, which include a polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof, may be administered to an animal. Such producer cells may, in one embodiment, be administered systemically (e.g., intravenously or intraarterially). The producer cell line then produces retroviral vectors including a polynucleotide comprising the polynucleotide encoding a sphingosine kinase, or an analogue, fragment, or derivative thereof.

Another embodiment has the expression of sphingosine kinase controlled by an inducible promoter. The use of an inducible gene expression system would allow the precise regulation of sphingosine kinase in a reversible manner. Several inducible systems are currently available. One example of a controlled promoter system in the Tet-On™ and Tet-Off ™systems currently available from Clontech (Palo Alto, CA). Tet-Off™ system uses the tetracycline-controlled transactivator (tTA), which is composed of the tet repressor protein (TetR) and the VP16 activation domain. tTA activates transcription in the absence of tetracycline. The Tet-On™ system uses the reverse tetracycline-controlled transactivator (rtTA) and activates transcription in the presence of tetracycline. Both systems use the tetracycline-response element (TRE), which contains 7 repeats of the tet operator sequence, and the target gene, such as sphingosine kinase. tTA or rtTA bind to the TRE, activating transcription of the target gene. This promoter system allows the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. This system could be used to control the expression of sphingosine kinase in this instant invention.

Other regulatable promoter systems are described in the US patent applications 09/586,625 and provisional application, number to be assigned, filed July 18, 2000, as Application No. 09/619,063 for "Regulation of Gene Expression Using Single-Chain, Monomeric, Ligand Dependent Polypeptide Switches" and subject to a petition for conversion to provisional application, filed July 18, 2001.

The expression vehicles, such as adenoviral vectors or retroviral vectors (including lentiviral vectors), or cells transduced with such expression vehicles, may be employed in the treatment of a variety of diseases or disorders. Such diseases and disorders include, but are not limited to, coronary artery disease, peripheral vascular disease, wound healing and fracture repair, reconstructive surgery, transplantation such as islet transplants, tendon

repair/sports injury, healing of ulcers, thromboangitis obliterans (Buerger's disease), periodontal tissue regeneration, and radiotherapy-induced esophagitis.

Contractile function loss in the heart is a major disease indication with 15 million worldwide and around 500,000 per year diagnosed with congestive heart failure (Massie, B. and Shah, N. American Heart Journal, 1997; 133:703-712). Congestive heart failure may be caused by myocardial ischemia and can be treated by therapeutic angiogenesis. Therapeutic angiogenesis can also replace current surgical interventions for the treatment of myocardial ischemia including percutaneous coronary intervention and coronary arterial bypass surgery. In addition, critical limb ischemia which develops in 500-1,000 people per million per year, often is not amenable to surgical or percutaneous revascularization and results in the loss of the limb. Again therapeutic angiogenesis can be used to treat this disease. A particular form of this disease is thromboangitis obliterans or Buerger's disease and preliminary clinical studies suggest an angiogenic approach may provide a novel therapy for patients with this disease. Sphingosine kinase-mediated angiogenesis can also be used to accelerate wound healing which requires a robust angiogenic response in granulation tissues following insults such as burns and to stimulate angiogenesis for better repair of bone fracture, bone grafts, and in healing tendons. Other possible use of this treatment include ulcer healing, periodontal tissue regeneration, reconstructive surgery and radiotherapy-induced esophagitis. Transplantation of encapsulated pancreatic islets is a promising treatment of type 1 diabetes and can greatly benefit from the use of sphingosine kinase to enhance vascularization (de Vos et al.., 1997).

For the treatment of congestive heart failure, myocardial ischemia, ischemia-reperfusion injury or peripheral arterial diseases, the lentiviral vector is administered to an animal in an effective amount from about 5×10^5 transducing units to about 10^{12} transducing units. In a preferred embodiment the effective amount delivered to the animal is from about 5×10^5 transducing units to about 10^{10} transducing units.

For the treatment of congestive heart failure, myocardial ischemia, ischemia-reperfusion injury or peripheral arterial diseases, the adenoviral vector is administered in an effective amount from about 10^7 plaque forming units to about 10^{12} plaque forming units, preferably from about 5×10^8 plaque forming units to about 2×10^{11} plaque forming units. Alternatively, cells transduced with the adenoviral vector may be administered in an amount of from about 10^3 to 10^8 cells.

EXAMPLES

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

EXAMPLE 1

Sphingosine Kinase Cloning and Southern Analysis

The plasmid pCR3.1sphK1α, derived from pCR3.1 (Invitrogen), was obtained from Thomas Baumruker (Novartis, Vienna, Austria) and contains the mouse sphingosine kinase alpha cDNA. pCR3.1sphK1x was digested with HindIII and NotI to isolate a 1,531 bp insert containing the coding sequence for sphK1α. The fragment was blunt-ended and cloned into the EcoRV site of pAVS6alx, an adenoviral shuttle plasmid containing a lox recombination site, to create pAV1xsphK1α (Figure 1). pAVS6alx had been formed by adding a lox site to pAVS6a (U.S. Patent No. 5,543,328). A 535 bp ClaI/NcoI fragment from pAVH8-101 lx, containing the SV40 polyA signal and lox site was inserted into pAVS6a digested with ClaI and NcoI and linearized (4,745 bp). The sphK1α cDNA was cloned downstream of the RSV promoter and the adenoviral tripartite leader sequence, and included the SV40 polyadenylation signal and a homologous recombination region. A large-scale plasmid preparation was prepared using the alkaline lysis method and purified using a CsTFA gradient following standard protocols. The cDNA then was sequenced. The sphK1α coding sequence is 1,149 bp (SEQ ID NO:5) and encodes a 382 amino acid protein (SEQ ID NO:6). The cDNA and amino acid sequences are shown in Figure 2.

Generation of a recombinant adenoviral vector

The sphk1α cDNA was incorporated into an adenoviral vector using the lox recombination three-plasmid transfection system. AE1-2a cells (Gorziglia et al., J. Virol., Vol. 70, No. 6, pgs. 4173-4178 (1996)), also known as S8 cells, were cultured in Richters media containing 5% heat inactivated fetal bovine serum (FBS). Transient transfections of the AE1-2a cells were performed with 0.5 μg of *NotI*-digested pAv1xsphk1α, 0.5 ug of pCcre and 1 μg of *ClaI*-digested pSQ3 DNA using the LipofectAMINE-PLUS reagent system (Life Technologies, Rockville, MD). Plasmid pSQ3 is a 31,574 bp plasmid containing adenoviral structural genes, but is devoid of E1, E2a, and E3 sequences. S8 cells were incubated with the lipofectamine reagent/DNA precipitate at 37^0C for 16 hours. The precipitate was removed and the monolayers were washed with PBS. Richters media containing 5% FBS and 0.3uM dexamethasone was added to the cells. The cells were

incubated at 37^0C for approximately 5-7 days. At that time the conditioned media and cells were collected, frozen and thawed three times and the cell debris was pelleted. The conditioned media was then used to infect a fresh plate of dexamethasone-induced S8 cells. A cytopathic effect was observed in the cells approximately 12-15 days post-transfection. The virus was amplified in 15 cm dishes of dexamethasone-induced S8 cells. The recombinant Av3sphk1α vector (Figure 3) was purified and a large scale preparation seedlot was prepared.

Southern Blot Analysis

The genomic organization and purity of Av3sphk1α was verified by DNA analysis of isolated viral DNA by restriction endonuclease digestion and Southern blot analysis following standard protocols using a [^{32}P]-labeled sphk1α cDNA probe. DNA was isolated from Av3nBg, described in published PCT Application No. WO96/18418, and Av3sphk1α and 1 μg of each was digested with *Kpn1* and *Sal1* and applied to a 0.8% agarose/TAE gel. Lanes 1-5 contain DNA molecular weight markers (Lane 1), Av3nBg and Av3sphk1α digested with *Kpn1* (Lanes 2 and 3, respectively) and Av3nBg and Av3sphk1a digested with *Sal1* (Lanes 4 and 5, respectively). The DNA fragments were transferred to a nylon membrane and prehybridized in 0.5M $NaPO_4$, 1mM EDTA, 0.5% BSA, 7% SDS at 65^0C for 2 hours. The membrane was hybridized at 65^0C overnight with the 1531 bp *Hind III/Not1*-digested fragment used for cloning into the shuttle plasmid and washed in SSC/SDS containing buffers at 65^0C following standard protocols. The membrane was exposed to film for 20 minutes at room temperature. The expected fragment derived from Av3sphk1α was detected by Southern Blot analysis (Data not shown).

EXAMPLE 2

Sphingosine kinase activity

The sphingosine kinase adenoviral vector was characterized first in vitro. Because immunological reagents currently are unavailable for this protein the ability of Av3sphk1α transduced cells to convert sphingosine to SPP was examined.

S8 cells, A549 cells (ATCC No. CCL-185), coronary artery smooth muscle cells (CASMC), or human umbilical vein endothelial cells (HUVEC), were transduced with Av3sphk1α or AV3Null at a multiplicity of infection (MOI) of 100, or were mock transduced.

2 days after transduction, cells were harvested and lysed via freeze-thawing in reaction buffer. Cell lysates were fractionated into cytosol and membrane fractions by centrifugation at 100,000x g for 60 min at 4° C. Sphingosine kinase activity was determined in the presence of 50 μM sphingosine (dissolved in 5% Triton X-100) , and [P] ^{32}ATP containing $MgCl_2$ (200 mM) and incubated for 15 min at 37°C.

Reactions were terminated by addition of 20 μl of 1N HCL,0.8 ml of chloroform/methanol/HCL(100:200:1, v/v), and 240 μl of chloroform and 240 μl of 2M KCL. Phases were separated by centrifugation. The labeled SPP in the organic phases was separated by thin layer chromatography on silica gel G60 with 1-butanol/ethanol/acetic acid/water (80:20:10:20 v:v) and visualized by autoradiography.

Mock transduced S8, A549, CASMC and HUVEC contained a very low level of sphingosine kinase enzymatic activity. Transduction of S8, A549, CASMC with an Av3Null vector does not substantially alter the amount of sphingosine kinase activity. By contrast, adenoviral transduction of HUVEC apparently increased endogenous sphingosine kinase activity. Transduction of all cell types with Av3sphk1α resulted in substantially higher enzymatic activity. The data indicate that the adenoviral vector encoding sphingosine kinase specifically increases the ability of cells to generate higher levels of SPP. These results indicate the vector expresses a functional sphingosine kinase enzyme that is capable of increasing cellular levels of SPP.

EXAMPLE 3

In vivo analysis using Matrigel implant assay

Samples including liquid Matrigel (a sterile extract of basement membrane proteins), admixed with test substances, are prepared beforehand and preloaded into 1.0 ml tuberulin syringes with G27 needles. Animals were anesthetized and 0.5 mls of the undiluted MatriGel containing 250 ng/ml or 1.2 ug per ml of FGF, adenoviral vectors or cells that are transduced with adenoviral vectors were injected subcutaneously into the caudal portion of the midline (1 injection/animal) using a G27 needle. The Matrigel will form rapidly a solid gel that persists for over 7 days. On Day 7, all animals are euthanized by CO_2 and Matrigel plugs are harvested for histological analysis and hemoglobin measurement. The tissues are stained for the endothelial cell specific marker, CD31 and for the smooth muscle cell marker, α smooth muscle actin.

To determine if Av3sphk1α induces angiogenesis in vivo, a matrigel implant model in athymic mice was used (Passaniti, et al, Lab. Invest., Vol. 67, No. 4, pgs. 519-528, 1992) S8

cells transduced with Av3sphk1α at 100 particles per cell are mixed with 0.5 Matrigel and implanted subcutaneously for 7 days. Av3sphk1α clearly enhances bFGF-induced angiogenesis as demonstrated by histological analysis (Data not shown). Appearance of larger, mature vessels as well as increased number of CD-31 positive vessel structure are apparent in the presence of Av3sphk1α transduced cells. By contrast, the control Av3null vector does not enhance angiogenesis. Next, whether direct incorporation of the Av3sphk1α vector into Matrigel is able to augment bFGF induced angiogenesis was examined. 1×10^9 total particles was mixed with 250 ng/ml bFGF and tested in the implant assay. As shown in Figure 4, direct vector incorporation facilitated increased angiogenic response as measured by an increase in the hemoglobin concentration in the Matrigel. The increased angiogenic response is similar to that produced by Av3sphk1α transduced cells. We conclude that overexpression of sphingosine kinase via an adenoviral vector can enhance blood vessel formation substantially *in vivo* and most strikingly, facilitated an increase in the size and maturity of the new vessels.

Figure 5 illustrates the remarkable differences that the addition of the adenoviral vector expressing sphingosine kinase makes in the nature of the neovessels that are formed in the *in vivo* matrigel system. As shown in Figure 5A, which is a hematoxylin and eosin stained section of matrigel treated with S8 cells transduced with the adenoviral vector encoding sphingosine kinase. There clearly is a vascular network including an arteriole and venule running side by side and a network of capillaries. In addition, these clearly are patent vessels as indicated by the presence of red blood cells, within these vessels. Figure 5B is the same section stained with α-smooth muscle (α-SM) actin. This section shows the presence of at least a single layer of actin positive cells surrounding the endothelium. By contrast, as shown in Figure 5C, a matrigel plug treated with FGF generated capillaries but there is a minimal amount of actin positive cells. Greater and more consistent α-SM actin staining in matrigel sections treated with the sphingosine kinase vector or cells transduced with this vector were observed routinely. The FGF-induced vessel had variable amounts of α-SM actin positive cells.

EXAMPLE 4

Sphingosine Kinase-Mediated Protection of Cardiomyocytes

Cell Lines and Culture Conditions: Human fetal cardiomyocytes (Clonetics Corporation, passages 4-7) were grown in 75 cm2 tissue culture flasks (Falcon Primaria) in Smooth Muscle Growth Media-2 (Clonetics Corporation) supplemented with 5% fetal bovine serum,

0.5 μg/ml human recombinant epidermal growth factor, 5 mg/ml insulin, 1 μg/ml human recombinant fibroblast growth factor, and 50 mg/ml gentamicin and 50 μg/ml amphotericin-B at 37° C in a 95% air/5% CO2 humidified atmosphere. Cells were subcultured by aspiration of the growth medium followed by a 30-sec rinse with a solution of 0.5 mM EDTA/0.25 mg/ml trypsin.

Apoptosis assays: Ischemia in cardiac myocyte cultures was induced by adding serum- and glucose-free Dulbecco's Modified Eagle Medium (Life Technologies) and incubating the cells in an incubator perfused with 95% N_2/5% CO_2. After the indicated time periods, the cells were removed from the hypoxic incubator, reoxygenated with complete growth medium containing glucose and serum and placed at 37°C in a 95% air/5%CO_2 humidified atmosphere. Cardiac myocytes were heat shocked by immersion into a temperature-controlled water bath (Precision Scientific, Chicago, IL) at 42°C for 30 min. Following exposure to heat shock, the cells were incubated at 37°C in a CO_2 incubator for 6 hr and then analyzed for apoptosis induction. Cells were treated with 10 μM C6-ceramide (N-Hexanoylsphingosine) overnight (Biomol Research Laboratories, Plymouth Meeting, PA) in serum-free medium prior to assessment for apoptosis. Cardiac myocytes were examined for morphological features of apoptosis (chromatin condensation and fragmentation) by fluorescence microscopy using acridine orange and ethidium bromide as described previously (Hreniuk, D., Garay, M, Gaarde, W., Monia, B.P., McKay, R.A. and Cioffi, C.L. Mol Pharmacol. 59: 867-874, 2001). Briefly, apoptosis was assessed by the addition of 50 μl of a 1:1 stock solution of ethidium bromide/acridine orange (Sigma) to 1 ml of culture media on the cells growing in 35 cm culture dishes. A coverslip was attached and the morphological features of apoptosis were monitored by fluorescence microscopy using a microscope equipped with FITC filter at 600X. At least 200 cells from randomly selected fields were counted and quantitated according to the following formula: % apoptotic cells = number of apoptotic cells/total number of cells counted x 100.

Adenovirus Transduction of Cardiac Myocytes: Transduction of human cardiac myocytes with adenoviral vectors was optimized with the adenoviral vector encoding the marker gene nuclear βgalactosidase (Av3ng). Briefly, cardiac myocytes, grown in 35 mm Primaria tissue culture dishes, were infected with adenoviral vectors at 10-500 particles per cell in 0.5 ml serum-free medium in the presence of a 1:1000 μl dilution of Fugene-6 for six hours at 37° C, and then 1 ml complete growth medium was added for overnight incubation. Cardiac

myocytes grown in 96-well Primaria culture plates were infected with Av3nBg in 100 μl serum-free medium for six hours at 37° C and then 100 μl of complete growth medium was added for overnight incubation.

Overexpression of Sphingosine Kinase protects cardiomyocytes from apoptosis: The AV3SK vector and its enzymatic product, sphingosine 1-phosphate (S-1-P) were both evaluated for possible inhibition of apoptosis in human cardiac myocytes induced by ceramide (n=3). heat shock (n=3), ischemia/reoxygenation (n=2). For vector-mediated studies, experiments were performed 3 days after vector treatment. The data, shown in Figure 6, indicate that S-1-P is a potent inhibitor of human cardiac myocyte cell death. Av3SK transduced cells are also almost completely resistant to heat shock and ischemia/reoxygenation-induced apoptosis (Fig. 6). However, Av3SK vectors can only partially inhibit ceramide-induced apoptosis. This data in cardiac myocytes supports a cardioprotective role for sphingosine kinase and S1P. The use of a gene therapy vector to express sphingosine kinase represents a treatment modality for the long-term protection of cardiac myocytes from injury and protect against congestive heart failure.

The disclosure of all patents, patent applications, publications (including published patent applications), depository accession numbers, and database accession numbers are incorporated herein, by reference, in their entirety to the same extent as if each individual patent, patent application, publication, depository accession number, and database accession number were specifically and individually incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.